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Optimization of RPLC Method for Separation of Some Acetylcholinesterase Inhibitors by using Central Composite Design

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ABSTRACT
This study is described for reversed phase liquid chromatographic separation of acetylcholinesterase inhibitors (donepezil, galantamine and rivastigmine). In the first stage of method development, acetonitrile concentration, pH of mobile phase and column temperature were investigated using central composite design (CCD). Afterwards, the optimal conditions were found employing central composite design and Derringer's desirability function. Effect of these variables on the output responses such as retention factors, resolutions (R_s) and retention time (t_R) were evaluated. The separation was applied by using X Terra C18 column (250 × 4.6 mm ID, 5 µm). The optimum assay conditions were: acetonitrile-water binary mixture (45:55, v/v) and pH 9.5 as the mobile phase and at column temperature 33 °C. Total chromatographic analysis time per sample was approximately 12.5 min. The method showed good agreement between the experimental data and predictive value throughout the studied parameter space. By using equations obtained CCD, protonation constant values (pK_a) of donepezil, galantamine and rivastigmine were also predicted.
Keywords: acetylcholinesterase inhibitors, optimization, pK_a estimation, central composite design (CCD), derringer's desirability function

INTRODUCTION

Alzheimer's disease (AD) is anoncoming, complicated neurodegenerative disease, which is the most common inducement of dementia in people excessive 60 years old. AD is characterized by progressive loss of neurons and synapses in the cerebral cortex. The reason

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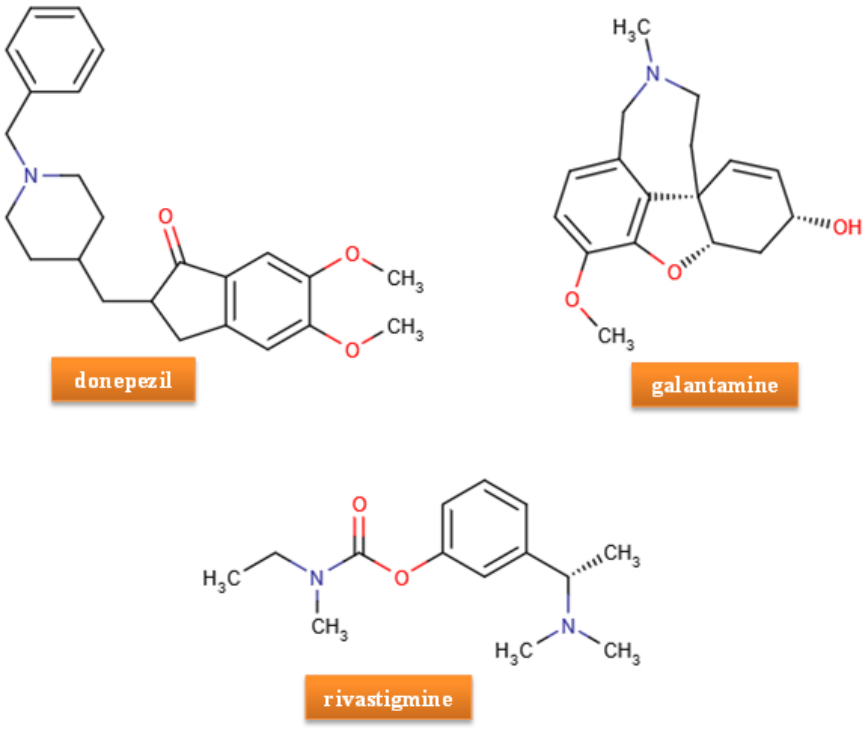

Figure 1. Molecular structures of donepezil, galantamine and rivastigmine

of the disease has not been completelyexpounded. The principal risk factor for AD is age because the sphere of influence of the disease increases considerably with age [1].

Cholinesterase inhibitors are the 'first-line' agents in the treatment of AD. Two cholinesterase enzymes are supply in the body, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The reversible AChE inhibitors that penetrate through the blood–brain barrier can be used in the theraphy of Alzheimer's disease. New approaches to determining the efficiency of cholinesterase inhibitors in the brain could include the use of several imaging techniques. The knowledge base for the pharmacodynamics and pharmacokinetics of cholinesterase inhibitors continues to enlarge. The enhanced information practical to clinicians can optimise the use of these agents in the management of patients with Alzheimer's disease [2].

Only four acetylcholinesterase inhibitors have been approved as anti-AD drugs. The first drug was tacrine. Currently, its use has been limited because of the high incidence of side effects, including hepatotoxicity, disorders of the gastrointestinal tract and hypotension. Other drugs used in AD include donepezil, rivastigmine and galantamine (**Figure 1**). These drugs are the basis for the treatment of Alzheimer's disease, and their effectiveness is supported by numerous studies. AChE inhibitors can slow the progression of AD, but not stop the disease [1, 3].

Generally, the trial and error approach based on the variation of one factor at a time is prefered to choose the best chromatographic conditions of drugs combination containing more than one active pharmaceutical ingredient (API). This approach, however, is consuming time and may not achieved the adequate separation due to the many variables and interactions involved. The resolution of a separation is of prime importance in reversed phase chromatography. In liquid chromatographic separations, resolution is influenced by composition of mobile phase and column temperature. The organic modifier concentration in the mobile phase would be a very effective tool to manipulate the resolution of a separation. In many instances, drugs include acidic and basic functional groups, therefore mobile phase pH is an important variable. On the other hand, the effect of temperature may be different enough for polar or ionisable compounds, selectivity changes associated to temperature are larger for this type drugs. The effect of three variables on resolution have been also investigated [4, 5].

The first step of chromatographic method development is to define the most favorable column, buffer and organic solvent that will be used to separate underresearched substances in a particular sample. When preliminary starting conditions have been found, the next step is to find the best separation conditions [6]. Before any optimization process, two significant parameters should be considered. Firstly a criterion should be defined for the evaluation and comparison of the results. The second parameter is the number of experiments. The best separation condition should be obtained with the minimum number of experiments. Besides, because of dependence on an extensive number of factors involving the percentage of organic modifier, mobile phase composition, temperature and flow rate, optimization of the chromatographic conditions is a complicated process in reversed phase liquid chromatography (RPLC). In addition to this, since the effect of these factors on retention can be interconnected and nonlinear the systematic approach for optimization of chromatographic separations is more convenient for such complicated method [7].

The method for the analysis of compound should be enough robust, sensitive and precise; hence, in this method design of experiment has been applied to study the effect of factors individually and in combination also. Experimental design is a process to organise the experiments properly to ensure that the right type of data, and enough of it will be available to answer the questions of interest as clearly and efficiently as possible. This design technique is based upon the principles of mathematical equations or models and outcomes of the factors [8].

In recent years, experimental design techniques have been often applied to change the resolution of separation methods [9-15]. The response surface methods (RSM) are very useful for interpreting the relationships between responses and factors. A central composite design (CCD) employing RSM can be used for optimization of the most important parameters by multiple regression models for response variables. CCD are normally used to determine which experimental factors are the most important to research and which factors do not substantially affect the experimental results. This type of design can be also used to estimate parameter

levels producing maximum or minimum response values and to optimize chromatographic parameters such as composition of mobile phase/column temperature. Many applications were made to form mathematical models for estimation of the retention factor of analytes in RPLC [16, 17].

CCD integrated with Derringer's desirability function (DF) is one of the most frequently used multi response optimization techniques in practice. A novel approach that can be used to attain the optimized separation condition of studied compounds in experimental design [18]. DF is a established technique to simultaneously determine of input variables that can give the optimum performance levels for one or more responses [19]. Using the DF, the overall desirability of the system in response to the controllable factors in the CCD could be optimized by transformation of different responses into one measurement. Therefore, the combination of CCD and DF may produces more efficient approach to predict or optimize multi-responses in a system by one experimental design [18].

The present manuscript describes the development, optimization and validation of an RPLC method for the routine quality control analysis of galantamine, rivastigmine and donepezil in a pharmaceutical laboratory. Among the pharmaceutical industries use time consuming LC method and various mobile phases for different dosage form of drugs. But with the proposed method developed, cost and time necessary for changing mobile phases could be saved, because only one mobile phase can be used for all the drugs and their combinations. Although, many methods for estimation of studied drugs individually have been reported in the literature, no single method is available for their simultaneous determination. Because use of these drugs is increasing rapidly, however, it is essential to develop a suitable analytical method for simultaneous estimation of galantamine, rivastigmine and donepezil in different preparations. Because RPLC method has been widely used for routine quality-control assessment of drugs, because of their sensitivity, repeatability, and specificity. Currently, there is no completely simultaneous optimization of organic solvent concentration, pH of the mobile phase and column temperature reported in the literature. A search of the literature revealed that a number of RPLC methods have been developed to determine the individual or several drugs in dosage forms [20-25] or in biological fluids [26-29]. The aim of this study is to improve a general method allowed the best separation of investigated compounds by means of CCD and desirability function and to predict values of retention factor by using equation obtained from experimental design at studied zone. Another aim of this paper is to compare protonation constant (pK_a) values obtained from experimental retention factor values with value of pK_a calculated from predicted values of retention factors.

EXPERIMENTAL

Apparatus

RPLC analysis was performed using a high performance liquid chromatograph (Shimadzu Technologies, Kyoto, Japan) coupled to a Diode Array Detector (SPD-M20A), a

pump (LC-20AD), a column oven (CTO-10AS VP) and a degasser system (DGU-20A3). pH measurements of the mobile phase were carried out with a Mettler Toledo MA 235 pH/ion analyzer (Schwerzenbach, Switzerland) using M-T combination pH electrode. For the standardization of potentiometric system according to the IUPAC rules [30], potassium hydrogen phthalate solution (0.05 mol kg^{-1}) was used.

Software

The analysis of variance (ANOVA) and response surface plots, evaluation of the results and statistical analysis were achieved with MINITAB 16 [31].

Chemicals and reagents

All chemicals and reagents were of analytical grade. Donepezil and galantamine were purchased from Sigma-Aldrich (USA). Rivastigmine was obtained from Novartis Pharm Inc (Istanbul, Turkey). Fluvoxamine was chosen as internal standard and purchased from Sigma-Aldrich (USA). HPLC grade acetonitrile (organic modifier), potassium hydrogen phthalate (standard buffer), sodium hydroxide, ammonia, ammonium bicarbonate, were employed from Merck (Darmstadt, Germany). Ortho-phosphoric acid (min. 85%) was obtained from Riedel-de Haen (Riedel-de Haen Germany). Tablet formulations of donepezil and rivastigmine (Doenza® 5 mg and Exelon®1.5 mg, respectively) were procured from local market.

Chromatographic conditions

In this study, mobile phases used were different proportions of acetonitrile. *o*-phosphoric acid and ammonium bicarbonate (NH_4CO_3) were used as buffer components. Liquid chromatography was performed on X Terra C_{18} (250 mm x 4.6 mm ID, 5 µm) column. CCD combined with DF was used to optimise the chromatographic variables that had an important influence on the response. Optimum condition was determined as 45:55 (v/v) with 25 mM NH_4CO_3 at pH 9. The ranges of column temperature used for the optimization were 28.296-36.705. The mobile phase was filtered through a 0.45 µm Nylon membrane filter (Millipore, Milford, MA, USA) and degassed by ultrasonication (Cole Palmer, Vernon Hills, USA) before usage. Detection was performed at 210 nm for all compounds.

Preparation of standard solutions

For the investigation of the influences of parameters on the response factors, solutions of the studied drugs were prepared carefully at a concentration of 100 µg mL^{-1}. Mobile phase was preferred as dissolution solvent. Similarly, stock solutions of two internal standard (IS) (5 µg mL^{-1}) was prepared in mobile phase. All solutions were preserved from light and were usedwithin 24 h at +4^{o}C in refrigerator in order to avoid decomposition.

Calculation of protonation constants

Throughout the studied parameter space, retention factor values were predicted by using MINITAB 16 programme. At each drug, different pH values were studied, spread over the pH range from 6.5 to 10.5. The protonation constant values (pK_a) of investigated compounds were estimated by the non-linear regression program NLREG [32].The effect of solvent composition in mobile phases was investigated at seven solvent levels (32.5%, 35%, 37.5%, 40%, 42.5%, 45% and 47.5%, v/v). Chromatographic measurements were done at 30 °C with an eluent flow rate of 1 mL min^{-1}.

Preparation of tablet samples

Ten tablets (Doenza® 5 mg and Exelon®1.5 mg) were weighed and finely powdered. An accurately weighed amount of the powder equivalent to one tablet was transferred into a 100 mL volumetric flask. Powder was accurately weighed and dissolved in 50 mL acetonitrile and sonicated for 15 min. The solution in the flask made up to volume with acetonitrile. An aliquot of the solution was filtered prior to injection. After filtration, adding the appropriate IS solution and diluting them with mobile phase to obtain the final solution. The amounts of donepezil and rivastigmine were calculated from the corresponding regression equations.

Preparation of samples in human urine

Blank urine samples were obtained from healthy volunteers. This was further used to prepare urine standards for the validation.4 mL of acetonitrile are added to 1 mL of human urine samples. Stocks of obtained drug-free solution was spiked with known quantities of galantamine, while the concentration of the internal standard was maintained at 3.0 µg mL^{-1}. The spiked solution was vortexed and filtered via to a 0.45 µM filter and each filtered sample was injected to the HPLC system. All urine samples were collected in polypropylene tubes and frozen at -20°C until analysis.

Method validation

Validation studies were conducted using the optimized assay conditions based on the principles of validation described with ICH guidelines. Analytical parameters, including, accuracy, precision, linearity, detection limit and quantitation limit were evaluated.

Calibration graphs of donepezil and rivastigmine analysis were represented by plotting the ratio of the peak area of the drug to that of IS versus the drug concentration. Calibration data for rivastigmine and donepezil were generated from calibration solutions which is prepared adding compounds in the concentration range of 1-6 µg mL^{-1}. Fluvoxamine was selected suitable internal standard for pharmaceutical analysis. Fluvoxamine (IS) concentration was fixed at 0.5 µg mL^{-1}. Calibration solution of galantamine were prepared by spiking urine with the appropriate amounts of stock solutions. Calibration data for galantamine were generated from calibration solutions which is prepared adding compound in the concentration range of range of 2-12 µg mL^{-1} into 5 mL of blank human urine.

Rivastigmine was selected suitable internal standard for human urine analysis. The rivastigmine (IS) concentration was fixed at 3.0 µg mL^{-1}.

In tablet anlaysis, precision study was performed to find out intra-day and inter-day variations in the estimation of donepezil and rivastigmine of different concentrations, with the proposed method. Intra-day and inter-day assay precision were determined by adding known amounts of galantamine to urine collected from healthy volunteers. Precision was calculated by determining the intra and inter-day percentage relative standard deviations (RSD).

Recovery experiments from tables show the reliability and suitability of the method. To check the accuracy of the improved methods and to study the intervention of formulation additives, analytical recovery studies were performed by internal standard method. From the quantity of donepezil and rivastigmine found, the percentage recovery was calculated. To keep an additional check on the accuracy of this developed method, recovery experiments were performed by adding the known amount of pure drug to pre-analyzed samples of human urine sample. Blank urine samples were spiked with two different concentrations of each substance and a constant level of an internal standard. These spiked urine samples were processed using the procedures described above for the calibration standard. The percentage recovery was calculated by comparing the concentration obtained from spiked samples with the actual added concentration. After five repeated experiments, the average recovery was calculated for galantamine.

RESULTS AND DISCUSSION

Similar chromatographic behaviour of donepezil, rivastigmine and galantamine make it possible the simultaneous separation of these substances in one chromatographic run. However, partially polar properties of galantamine and extreme retention time of donepezil cause to inappropriate chromatographic separation. Due to this situation, it was required to evaluate the chromatographic behaviour of investigated drugs towards various parameters to describe the chromatographic conditions which would allow adequate separation of all compounds within the minimum possible analysis run time.

Screening based on a central composite design

The combined effect of eluent pH, column temperature and organic modifier content on retention behaviour of galantamine, rivastigmine and donepezil were investigated using central composite design. For three tested parameters, these designs require fourteen different runs, plus central point replications (6 experiments). Experiments were carried out according to the design listed in **Table 1**. The upper and lower limits of the factors have been determined from the preliminary experiments.

Table 1. Factors and their corresponding levels as central composite design

Independent Factors	(-α)	Low level (-)	Zero level (0)	High level (+)	(+α)
(X_1) acetonitrile (%)	31.591	35	40	45	48.409
(X_2) pH of the mobile phase	4.807	6	7.75	9.5	10.693
(X_3) Temperature (°C)	28.296	30	32.5	35	36.705

The data obtained by using CCD under different experimental conditions were correlated to the three variables and their interactions (MINITAB 16, USA). The design fitted with a full quadratic model is given below:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \qquad (1)$$

where the response function is presented with y. In the multiple regression, b_0 is the intercept; x_1, x_2, x_3 are the main effects; $b_1, b_2, b_3, b_{12}, b_{13}, b_{23}, b_{11}, b_{22}, b_{33}$are the regression coefficients of the model;x_1x_2, x_1x_3, x_2x_3 are the two - way interactions between the main effects. The coefficients of this quadratic equation were calculated by using MINITAB 16 programme. In this calculation process, resolution factor and logarithm of retention factor were selected as outcome of the experiments. The resolution factors were calculated by using Purnell equation. Before starting an optimization procedure, ANOVA was generated for factorial design shows that curvature is significant for the variable since p-value is less than 0.05. This implies that a quadratic model should be considered to model the separation process, such as a CCD to be employed for the subsequent optimization state.

Based on the results of the performed experiments the following polynomial equations were obtained:

$$\log k = -1.174 - 0.017x_1 + 0.566x_2 - 0.021x_2^2 - 0.003x_1x_2$$

where $\log k$ is optimized response value defined as logarithm of retention factor for galantamine

$$\log k = -5.767 - 0.039x_1 + 0.822x_2 + 0.080x_3 - 0.001x_1^2 - 0.032x_2^2 - 0.001x_3^2 - 0.002x_1x_2$$

where $\log k$ is optimized response value defined as logarithm of retention factor for rivastigmine.

$$\log k = -2.327 - 0.049x_1 + 0.715x_2 + 0.042x_3 - 0.001x_1^2 - 0.016x_2^2 - 0.001x_3^2 - 0.005x_1x_2$$

where log k is optimized response value defined as logarithm of retention factor for donepezil.

$$Rs_{2,1} = 3.330 - 0.980x_1 + 2.917x_2 + 0.929x_2^2$$

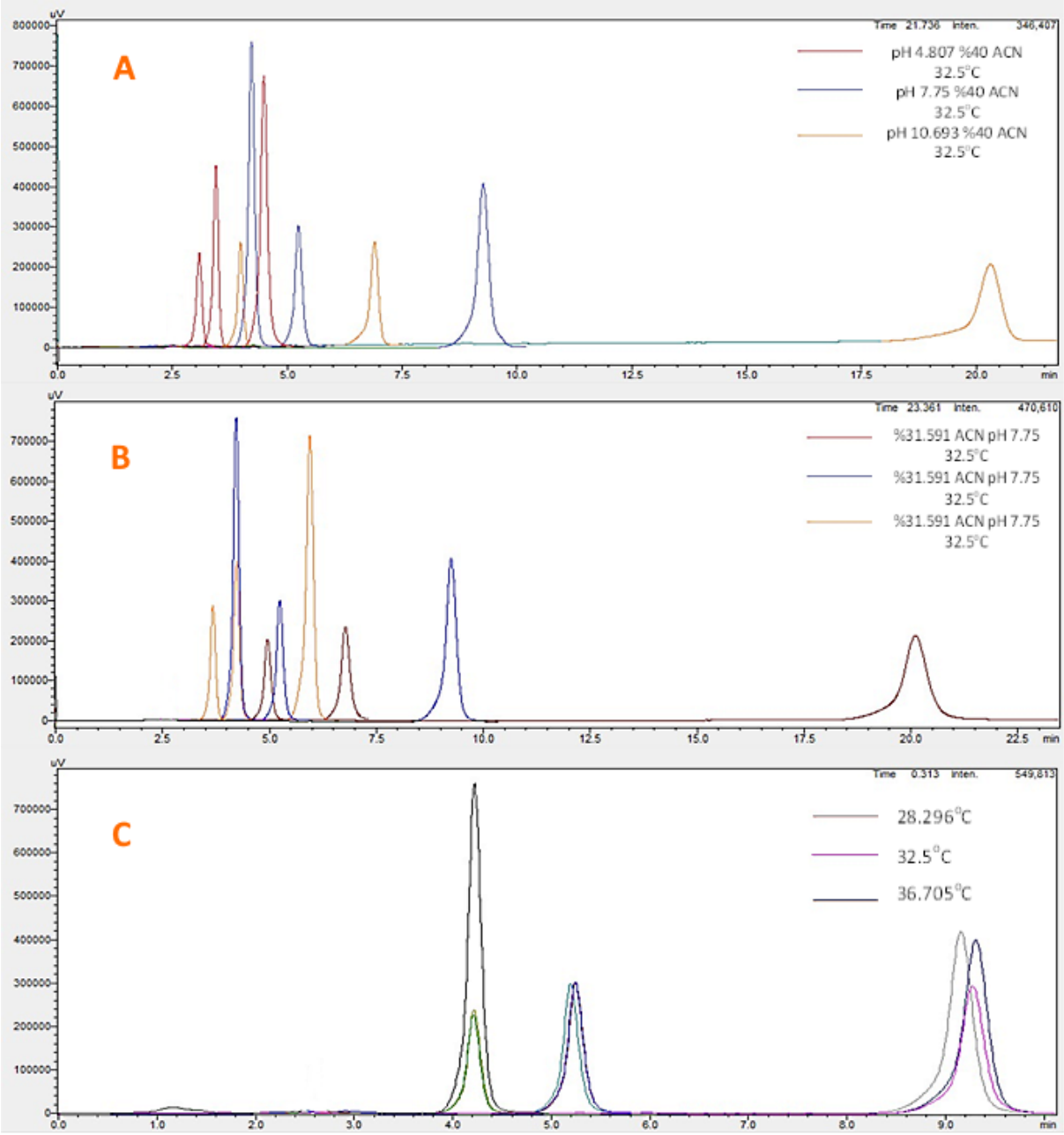

Figure 2. RPLC chromatograms for effects of organic modifier, pH of mobile phase and column temperature

where Rs is optimized response value calculated with Purnell equations from retention factor for rivastigmine/galantamine

$$Rs_{3,2} = 8.685 - 2.249x_1 + 4.339x_2 + 0.941x_1x_2$$

Table 2. Criteria for the optimization of the individual responses

Compound	Response	Goal	Lower limit	Upper limit	Target	Weight
Galantamine	k_1	Target	0.006	0.809	0.6	1
	$R_{s_{2,1}}$	Target	1.353	10.591	2	1
Rivastigmine	k_2	Target	0.086	2.525	1.2	1
	$R_{s_{3,2}}$	Target	3.000	17.548	3	1
Donepezil	k_3	Target	0.227	9.610	2.5	1

where R_s is optimized response value calculated with Purnell equations from retention factor for galantamine/donepezil.

x_1, x_2 and x_3 values given in equations is denoted the percentage of acetonitrile in the mobile phase, pH of mobile phase and column temperature, respectively. These equations is included only significant coefficients with $p < 0.05$. While x_1 and x_2 parameters are significant effect on log k and R_s values of drugs, x_3 parameters is unsignificant effect on $log\ k$ for galantamine and on R_s values for all drugs. Chromatograms of three effects were shown **Figure 2.**

The method optimization by desirability approach

The evaluation of each parameter individually on the response factors (retention times and resolutions) is very difficult when several responses exist in an experimental design. In the present study, the identified criteria for the optimization were logarithmic retention and resolution factors. Derringer's desirability function [33] was used to optimize two responses with different targets. The Derringer's desirability function, D, is defined as the geometric mean, weighted or otherwise of the individual desirability functions. Desirability function can take values from 0 to 1. Weights can range from 0.1 to 10. A value of D close to 1, indicates that the combination of the different criteria is matched in a global optimum. The criteria for the optimization of each individual response were shown in Table 2.

As can be seen under criteria, k_1 was targeted at 0.60 to be adequately high allowing complete separation of galantamine from the solvent peak. $R_{s_{2,1}}$ was targeted at 2.00 to permit baseline separation of galantamine and rivastigmine. $R_{s_{3,2}}$ was targeted at 2.00, for shorten the analysis time and to provide complete separation of rivastigmine and donepezil. k_3 was targeted at 2.5 to be as close as possible minimum capacity factor value. The weights for all the five responses (pi values) were adjusted at 1 and the resolutions and retention times obtained from experiments were converted to a desirability scale to get minimum retention time and maximum resolution. According to these values, the desirability of resolutions + retention factors (D_{All}), retention factors (D_{logk}) and desirability of resolutions (D_{R_S}) were calculated (**Table 3**).

Table 3. Experimental conditions according to the central composite design and total desirability function calculated from the responses

Exp No.	x_1	x_2	x_3	D_{logk} Galantamine	D_{logk} Rivastigmine	D_{logk} Donepezil	D_{R_s} Rivastigmine/ galantamine	D_{R_s} Donepezil/ rivastigmine	D_{All}
1	-	-	-	0.566	0.194	0.359	0.969	0.730	0.489
2	+	-	-	0.260	0.144	0.226	0.316	0.894	0.299
3	-	+	-	0.000	0.174	0.009	0.143	0.057	0.000
4	+	+	-	0.874	0.916	0.846	0.512	0.463	0.694
5	-	-	+	0.562	0.205	0.410	0.966	0.733	0.507
6	+	-	+	0.269	0.184	0.287	0.281	0.906	0.325
7	-	+	+	0.036	0.201	0.019	0.148	0.048	0.062
8	+	+	+	0.874	0.924	0.853	0.522	0.463	0.698
9	-α	0	0	0.405	0.847	0.453	0.554	0.289	0.477
10	+α	0	0	0.561	0.309	0.407	0.938	0.775	0.552
11	0	-α	0	0.000	0.000	0.000	0.000	0.990	0.000
12	0	+α	0	0.572	0.000	0.000	0.000	0.051	0.000
13	0	0	-α	0.856	0.715	0.908	0.000	0.562	0.000
14	0	0	+α	0.852	0.715	0.934	0.759	0.564	0.754
15	0	0	0	0.867	0.717	0.927	0.845	0.570	0.774
16	0	0	0	0.867	0.717	0.927	0.845	0.570	0.774
17	0	0	0	0.867	0.717	0.927	0.845	0.570	0.774
18	0	0	0	0.867	0.717	0.927	0.845	0.570	0.774
19	0	0	0	0.867	0.717	0.927	0.845	0.570	0.774
20	0	0	0	0.867	0.717	0.927	0.845	0.570	0.774

D_{R_s}: Desirability of resolutions, $D_{\log k}$: Desirability of logarithmic retention factors and D_{All}: Desirability of resolutions and retention factors

Value of D different to zero implies that all responses are in a desirable range simultaneously and consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so as the response values are near target values.

The response surface graphs were depicted as a function of ACN % (v/v) and pH; ACN % (v/v) and t (°C); pH and t (°C) (**Figure 3**). These response surface graphs permitted the determination of an optimal region, where the best separation could be established.

From the **Figure 3**, it can be conclude that optimum chromatographic conditions were selected by total desirability as near as 1.00. The highest desirability (around 0.967) was obtained when the acetonitrile level in the mobile phase was 45% (v/v) , the mobile phase of pH was pH 9.5 and column temperature was 33oC with an eluent flow rate of 1 mL min^{-1}. A representative chromatogram of developed method via to CCD combined with DF was given **Figure 4**.

Assay of method validation

In order to demonstrate improved analytical method, validation was carried out the guidelines given by theInternational Conference on Harmonization [34]. In this way, recovery,

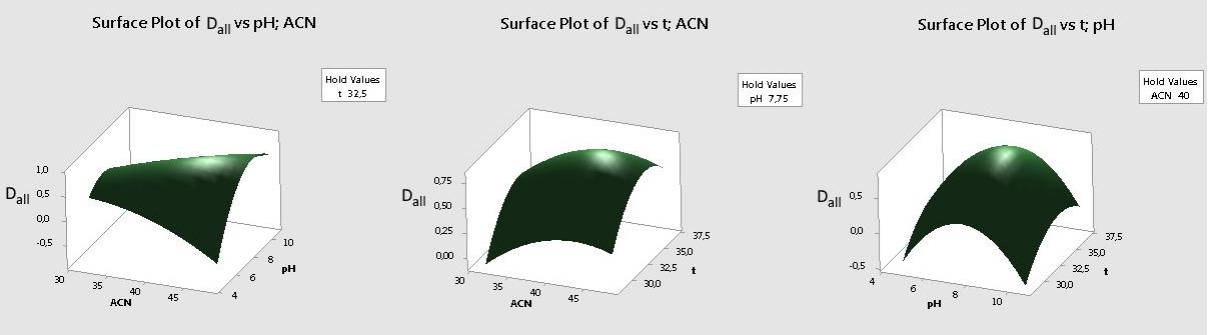

Figure 3. Graphical representation of the overall desirability function D

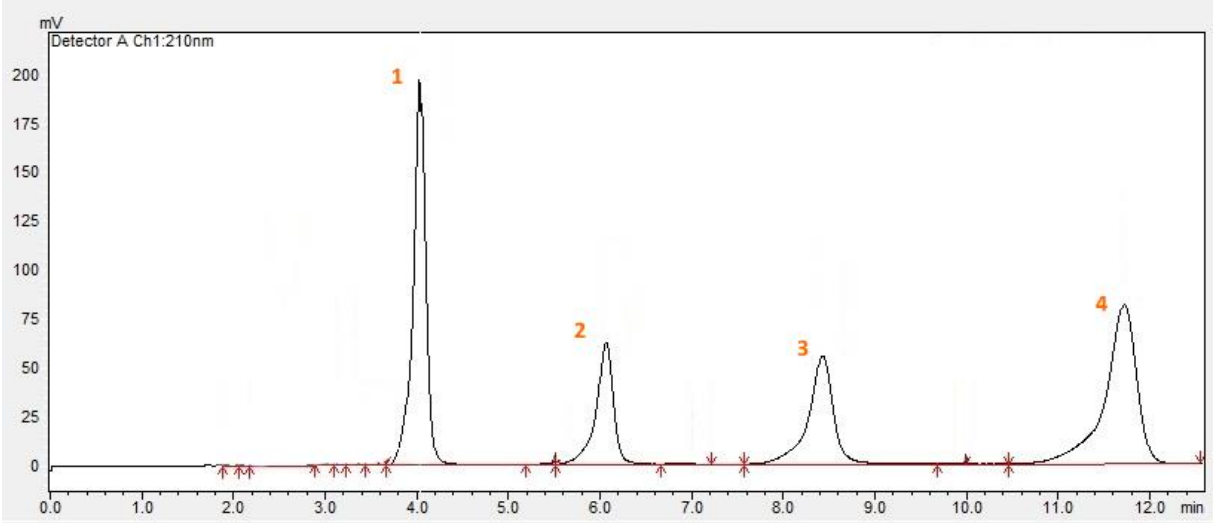

Figure 4. Chromatogram obtained at standard mixture. Galantamine (1), rivastigmine (2), fluvoxamine (IS) (3) and donepezil (4)

linearity, working range, intra- and inter- assay, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) were tested for galantamine, rivastigmine and donepezil.

Calibration and linearity

The linear range of the calibration curves for galantamine, rivastigmine and donepezil were determined over six concentration levels (**Table 4**). The equation acquired for each analyte with their correlation coefficient (r= 0.999) of determination demonstrated the good linearity at the concentrations ranging from 1 to 6 µg mL^{-1} for rivastigmine and donepezil, at the concentrations ranging from 2 to 12 µg $mL^{-1}$for galantamin.

LOD and LOQ

LOD and LOQ were calculated using the signal-to-noise method. Signal-to-noise is frequently used to estimate the LOD and LOQ in HPLC as it is easy to implement. The results of the LOD and LOQ were listed in **Table 4**, showing that the diode array detector is sensitive and that the improved method is accurate at low concentrations.

Table 4. Statistical evalution of the calibration data of galantamine, rivastigmine and donepezil by RP-LC

Sample	Linearity Range (µg mL^{-1})	Slope	Intercept	S.E. of Slope	S.E. of Intercept	Correl Coeff.	Detection Limit (µg mL^{-1})	Quantitation Limit (µg mL^{-1})
Galantamine	2-12	0.998	-0.071	0.007	0.056	0.999	0.197	0.597
Rivastigmine	1-6	2.198	0.112	0.031	0.121	0.999	0.196	0.593
Donepezil	1-6	6.147	0.119	0.056	0.216	0.999	0.125	0.378

Table 5. Accuracy and precision of the method for RPLC analysis of galantamine in human urine

Compound	Theoretical Concentration (µg mL^{-1})	N	Mean found concentration (concentration mean (µgmL^{-1}) ± confidence interval)	Recovery (%) (percentage mean ± confidence interval)	Standard Deviation	RSD (%)
Galantamine	4	3	4.074 ± 0.096	101.892 ± 1.955	0.787	0.773
	8	3	7.997 ± 0.088	99.960±0.899	0.362	0.362

Table 6. Results of the assay and the recovery analysis of rivastigmine and donepezil in pharmaceutical dosage forms

Parameter	Rivastigmine	Donepezil
Labeled claim (mg)	1.500	5.000
Amount found (mg)	1.497	5.029
RSD%	0.455	0.847
Bias(%)	0.222	-0.583
Added(mg)	1.5	5.000
Found (mg)[a]	1.494	5.034
Recovery %	99.572±1.065	100.688±1.736
RSD% of recovery	0.430	0.694
Bias%	0.428	-0.688

System suitability

System suitability tests are carried out to confirm that reproducibility of the chromatographic system and the resolution are sufficient for the analysis to be done. These tests were performed on freshly prepared standard stock solutions of galantamine/rivastigmine(IS) and rivastigmine, donepezil, fluvoxamine (IS). The parameters include retention time, theoretical plate number, retention factor, RSD (%) of of peak height or area for repeated injections. The theoretical plate numbers (N) were 5101 for galantamine, 7214 for rivastigmine (IS) (used for galantamine analysis), 6859 for rivastigmine, 8398 for rivastigmine and 7524 for fluvoxamine (IS) (used for rivastigmine and donepezil analysis). The selectivity factors were 2.544 for rivastigmine (IS)/galantamine, 1.733 for fluvoxamine (IS)/rivastigmine and 1.578 for donepezil/fluvoxamine (IS). The defined chromatographic conditions provided sufficient retention and resolution for all of studied analytes. The retention times of galantamine and rivastigmine (IS) were 4.098 and 6.231 min. respectively.

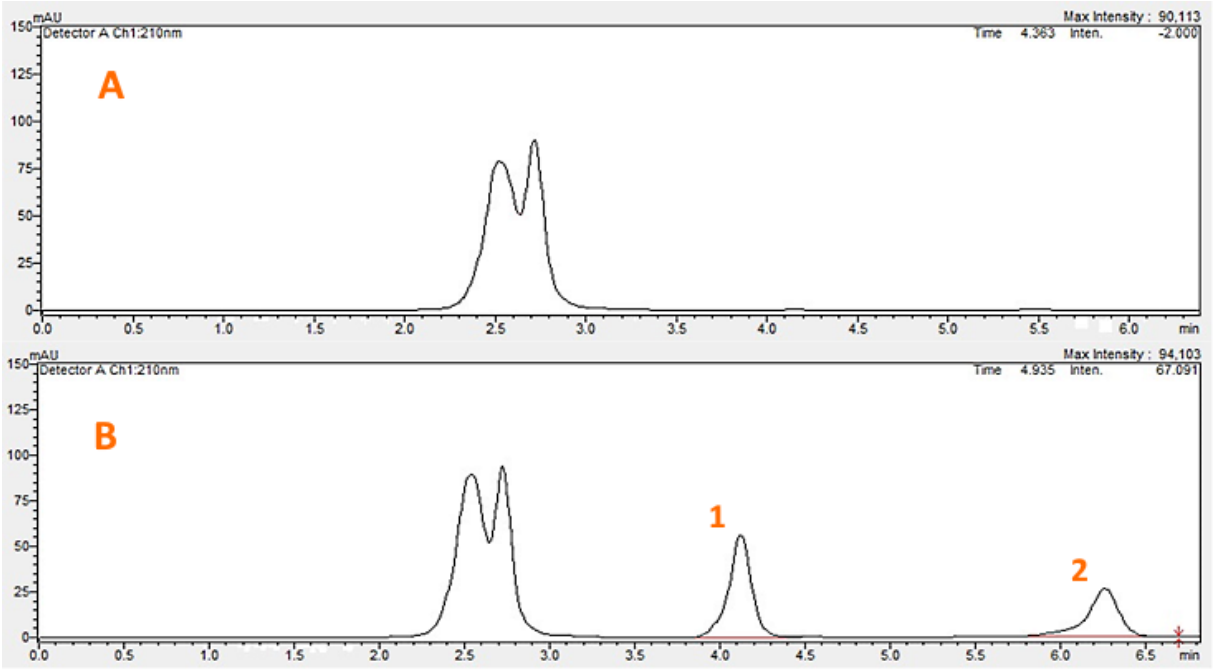

Figure 5. Typical chromatograms for blank human urine (A), human urine spiked with (1) galantamine at 4 μg mL^{-1} and (2) rivastigmine (IS) at 3 μg mL^{-1} (B)

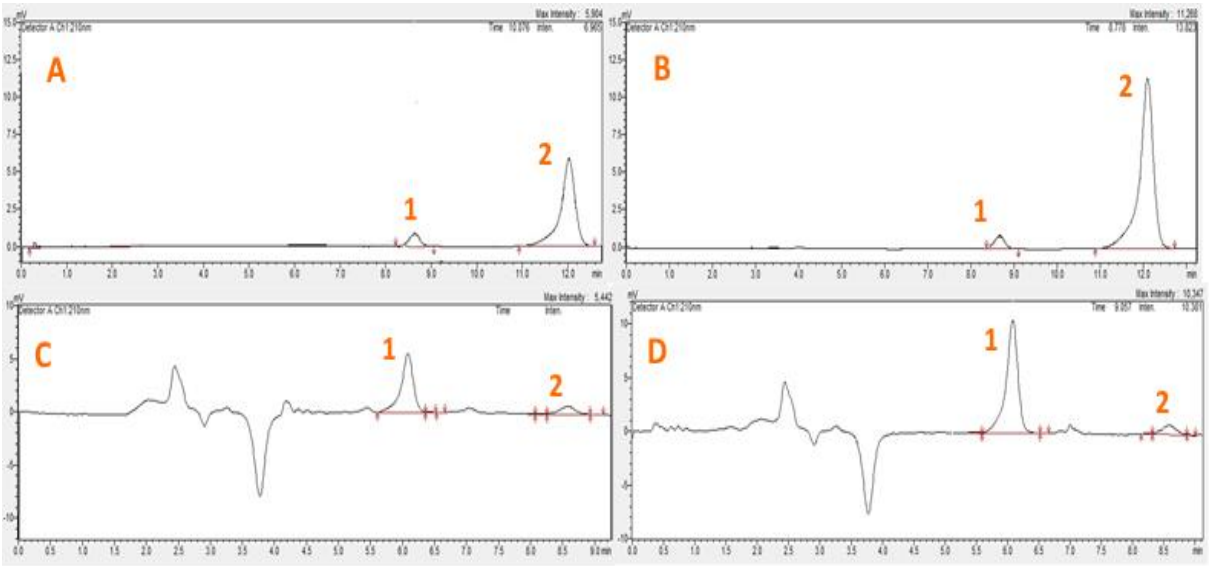

Figure 6. Chromatograms of donepezil, rivastigmine and fluvoxamine (IS) in pharmaceutical dosage forms. (A), 1. Fluvoxamine (IS) (0.5 μg mL^{-1}) ; 2. Donepezil (2 μg mL^{-1}); (B), 1. Fluvoxamine (IS) (0.5 μg mL^{-1}); 2. Donepezil (4 μg mL^{-1}); (C), 1. Rivastigmine (2 μg mL^{-1}); 2. Fluvoxamine (IS) (0.5 μg mL^{-1}); (D), 1.Rivastigmine (4 μg mL^{-1}); 2. Fluvoxamine (IS) (0.5 μg mL^{-1})

Table 7. Coded factor levels for CCD. Experimental k and predicted k values for studied compounds

Parameters									
Exp. No	ACN (%)	pH	Column temp (°C)	Exp. k values	Pre. k values	Exp. k values	Pre. k values	Exp. k values	Pre. k values
				Galantamine		Rivastigmine		Donepezil	
1	-1	-1	-1	0.335	0.341	0.315	0.316	1.041	1.042
2	1	-1	-1	0.155	0.159	0.192	0.193	0.726	0.739
3	-1	1	-1	0.809	0.808	2.520	2.525	9.543	9.543
4	1	1	-1	0.525	0.524	1.341	1.346	3.589	3.593
5	-1	-1	1	0.333	0.338	0.314	0.315	1.155	1.158
6	1	-1	1	0.160	0.164	0.190	0.190	0.871	0.876
7	-1	1	1	0.800	0.800	2.476	2.486	9.482	9.475
8	1	1	1	0.525	0.523	1.315	1.318	3.540	3.546
9	-α	0	0	0.730	0.723	1.297	1.291	6.390	6.390
10	+α	0	0	0.342	0.337	0.503	0.499	1.169	1.150
11	0	-α	0	0.006	0.007	0.087	0.086	0.227	0.212
12	0	+α	0	0.687	0.688	2.533	2.516	9.610	9.606
13	0	0	-α	0.519	0.513	0.860	0.854	2.300	2.288
14	0	0	+α	0.517	0.510	0.839	0.835	2.353	2.347
15	0	0	0	0.528	0.519	0.885	0.886	2.335	2.332
16	0	0	0	0.521	0.519	0.882	0.886	2.324	2.332
17	0	0	0	0.523	0.519	0.882	0.886	2.323	2.332
18	0	0	0	0.517	0.519	0.884	0.886	2.326	2.332
19	0	0	0	0.517	0.519	0.884	0.886	2.333	2.332
20	0	0	0	0.518	0.519	0.904	0.886	2.361	2.332

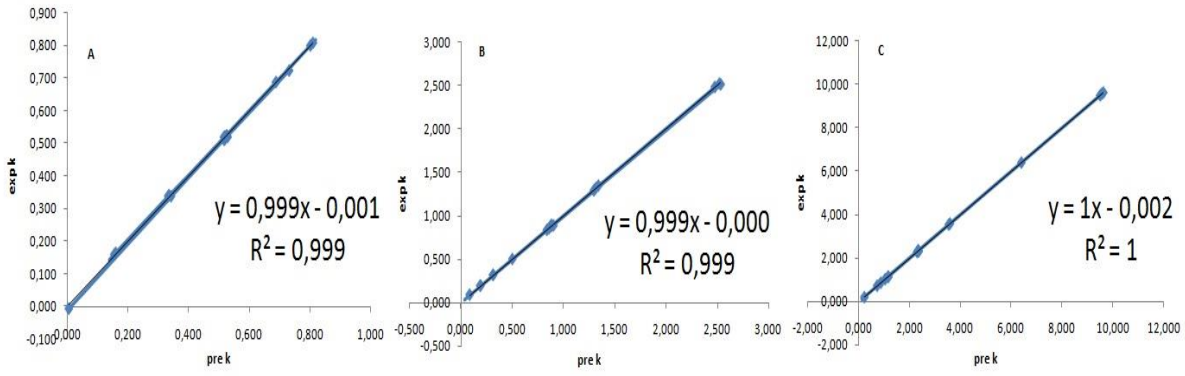

Figure 7. A plot of calculated k values versus measured k values of galantamine (A), rivastigmine (B), donepezil (C)

The retention times of rivastigmine, fluvoxamine (IS) and donepezil were 6.075, 8.571 and 11.900 min. respectively. The variation in retention time for three replicate injections of all compounds in reference solutions gave RSDs of 0.204% for rivastigmine, 0.493% for fluvoxamine (IS), 0.124% for donepezil and 0.112% for galantamine and 0.025% for rivastigmine (IS).

Precision and Accuracy

The precision of the proposed method for galantamine and rivastigmine (IS) was performed by adding known amounts of studied drugs into human urine with three replicate injections of galantamine and rivastigmine (IS) in different days. The precision of the proposed method for rivastigmine, donepezil and fluvoxamine (IS) were evaluated by performing replicate analysis (three replicates) of their standard solutions in different days. Within calibration curves, two different concentrations were prepared in both media and assayed with related calibration curves to determine inter-day and intra-day variability. The inter-day and intra-day precision, accuracy, and reproducibility were determined as the RSD% and mean value. Repeatability was calculated by assaying three samples of each at two different concentration levels (2 and 5 µg mL^{-1} for donepezil and rivastigmine, 4 and 10 µg mL^{-1} for galantamine) on the same day. The inter-day precision was calculated by assaying three samples of each at two different concentration levels (2 and 5 µg mL^{-1} for donepezil and rivastigmine, 4 and 10 µg mL^{-1} for galantamine) on three different days. The RSD range was obtained as 0.10-0.90 and 0.50-1.12 for intra-day and inter-day precision studies respectively. Precision, accuracy and reproducibility results demonstrate good precision,accuracy, and reproducibility.

The accuracy of the method for galantamine was determined with recovery experiments in human urine because of the fact that there is no tablet form of galantamine in Turkey. The accuracy of the method for rivastigmine and donepezil was determined with recovery experiments in commercial pharmaceutical dosage forms. The recoveries and RSDs after three replicate analyses are given in **Table 5** (for galantamine) and in **Table 6** (for rivastigmine and donepezil). **Figure 5** shows (A) the chromatogram attained for drug-free human urine in the enhanced chromatographic conditions and (B) the chromatogram attained for urine spiked with different concentrations of studied compounds.Chromatograms obtained from pharmaceutical dosage form samples (with IS) are shown in **Figure 6**. As $R_s \geq 1.5$-2.0 is generally accepted as a good resolution between the peak and the closest electing potential interference.

Determination of predicted protonation constant using central composite design

Prediction of retention factors with studied parameters was calculated by using the equations obtained from CCD (**Table 7**).

As shown in **Table 7**, the proposed method demonstrated well prediction ability between the experimental data and predictive values throughout the studied parameter space.

Table 8. Predicted retention parameters calculated by using central composite design and estimated pK_a obtained from NLREG programme

CAN (v/v) %	Galantamine	30 °C	Rivastigmine	30°C	Donepezil	30 °C
32.5%	pK_a	7.832±0.156	pK_a	8.496±0.153	pK_a	8.851±0.143
	k_{BH^+}	0.510±0.031	k_{BH^+}	0.448±(0.124)	k_{BH^+}	2.008±2.579
	k_B	0.898±0.017	k_B	3.353±0.186	k_B	20.514±1.527
35%	pK_a	7.795±0.151	pK_a	8.464±0.153	pK_a	8.799±0.147
	k_{BH^+}	0.451±0.029	k_{BH^+}	0.404±0.111	k_{BH^+}	1.527±0.425
	k_B	0.819±0.016	k_B	2.965±0.163	k_B	14.442±1.064
37.5%	pK_a	7.751±0.147	pK_a	8.432±0.154	pK_a	8.744±0.150
	k_{BH^+}	0.396±0.027	k_{BH^+}	0.360±0.097	k_{BH^+}	1.182±0.317
	k_B	0.742±0.014	k_B	2.576±0.140	k_B	10.342±0.753
40%	pK_a	7.702±0.180	pK_a	8.399±0.154	pK_a	8.688±0.154
	k_{BH^+}	0.621±0.108	k_{BH^+}	0.314±0.083	k_{BH^+}	0.931±0.240
	k_B	2.502±0.154	k_B	2.200±0.118	k_B	7.533±0.541
42.5%	pK_a	7.655±0.132	pK_a	8.365±0.154	pK_a	8.628±0.157
	k_{BH^+}	0.289±0.022	k_{BH^+}	0.270±0.070	k_{BH^+}	0.747±0.185
	k_B	0.594±0.010	k_B	1.846±0.090	k_B	5.582±0.397
45%	pK_a	7.588±0.123	pK_a	8.330±0.154	pK_a	8.566±0.161
	k_{BH^+}	0.237±0.020	k_{BH^+}	0.227±0.058	k_{BH^+}	0.609±0.144
	k_B	0.523±0.009	k_B	1.522±0.079	k_B	4.208±0.292
47.5%	pK_a	7.529±0.113	pK_a	8.295±0.154	pK_a	8.500±0.164
	k_{BH^+}	0.189±0.018	k_{BH^+}	0.188±0.047	k_{BH^+}	0.506±0.114
	k_B	0.455±0.007	k_B	1.234±0.063	k_B	3.226±0.220

Table 9. Aqueous pK_a values of galantamine, rivastigmine and donepezil obtained from two different methodologies

Compounds	Yasuda-Shed.	pK_a-X	Literature value
Galantamine	8.269	8.340	8.571 pK_a-X [36]; 8.21[37] 8.493 Yasuda-Shed. [36]
Rivastigmine	8.771	9.085	9.035 pK_a-X[36] 9.019 Yasuda-Shed. [36]; 8.90[38];8.99[39]
Donepezil	8.892	8.555	8.555 pK_a-X[36]; 9.10 [40] 8.534 Yasuda-Shed. [36]

A plot of calculated k values versus measured k is shown in **Figure 7**. The correlation is reasonably good in which the slope is unity and the intercept is nearly zero. This demonstrates that effect of chosen parameters on retention time can be predicted in the studied parameter space.

Because it is not always convenient or practical to carry out experimental measurements for pK_a determination, it is useful to improve easy-to-use and accurate models to estimate pK_a values for compounds. In this study, the pK_a values of of the investigated drugs were calculated by using the relationships between estimated k values and pH of the eluent with NLREG program (**Table 8**).

It is known that determination of aqueous dissociation constant is required for routine drug analysis. In present experiment, pK_a values of studied compounds were determined in the organic modifier-rich region of acetonitrile-water mixtures. There are two approaches to estimate of the pK_a in aqueous media from the mobile phase pK_a values. In the first approach, pK_a values are plotted versus acetonitrile mole fraction. The intercepts of these linear equations achieved from this approach are theaqueous pK_a valuesof these compounds (**Table 9**). In second approach, to forecast aqueous pK_a values from the pK_a values, Yasuda-Shedlovsky equation [35] is used (**Table 9**). A comparison with representative dissociation constants available in the literature shows that either method gives satisfactory values.

CONCLUSION

The present method was simultaneous procedure, which was very advantageous compared to the previously reported HPLC methods. This work represents the first study dealing with concurrent chromatographic determination and prediction of protonation constant of some acetylcholinesterase inhibitors. With assistance of central composite design (CCD) and Derringer's desirability function new chromatographic method has been developed. On the basis of predefined response targets, and by the adjustment of importance coefficients and weights, the optimal mobile phase composition was predicted. Being accurate, precise, sensitive and rapid this method is suitable for rapid screening of donepezil, rivastigmine and galantamine concentrations in pharmaceutical formulations and human urine samples. The validation of the assay is adequate in terms of generally accepted ICH guidelines for linearity, accuracy and precision. The method optimization was enabled investigate and determination of chromatographic behavior of the investigated compounds. Additionally, The CCD was utilised to predict RPLC retention factors of investigated substances by using equations obtained from CCD. The protonation constant values (pK_a) of investigated compounds were estimated by the non-linear regression program NLREG. pK_a values obtained from experimental retention factor values were compared pK_a calculated from predicted values of retention factors.

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